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Abstract: BACKGROUND Diagnosis of Cushing syndrome requires a multistep process that includes verification of hypercortisolism followed by identification of the cause of adrenocortical hyperfunction. This study assessed whether pituitary, ectopic, and adrenal subtypes of Cushing syndrome were characterized by distinct plasma steroid profiles that might assist diagnosis. METHODS In this retrospective cross-sectional study, mass spectrometric measurements of a panel of 15 plasma steroids were applied to 222 patients tested for Cushing syndrome. Disease was excluded in 138 and confirmed in 51 patients with pituitary Cushing syndrome, 12 with ectopic adrenocorticotropin secretion, and 21 with adrenal disease. Another 277 age- and sex-matched hypertensive and normotensive volunteers were included for comparison. RESULTS Compared with patients without disease, the largest increases in plasma steroids among patients with Cushing syndrome were observed for 11-deoxycortisol (289%), 21-deoxycortisol (150%), 11-deoxycorticosterone (133%), corticosterone (124%), and cortisol (122%). Patients with ectopic disease showed the most prominent increases, but there was considerable variation for other steroids according to subtype. Patients with adrenal disease had the lowest concentrations of androgens, whereas those with ectopic and pituitary disease showed the lowest concentrations of aldosterone. Plasma 18-oxocortisol was particularly low in ectopic disease. With the use of 10 selected steroids, subjects with and without different Cushing syndrome subtypes could be discriminated nearly as closely as with the use of salivary and urinary free cortisol, dexamethasone-suppressed cortisol, and plasma adrenocorticotropin (9.5% vs 5.8% misclassification). CONCLUSIONS Patients with different subtypes of Cushing syndrome show distinctive plasma steroid profiles that may offer a supplementary single-test alternative for screening purposes.

DOI: https://doi.org/10.1373/clinchem.2017.282582

Clinical Chemistry 64:3
000–000 (2018)

Endocrinology and Metabolism

Plasma Steroid Metabolome for Diagnosis and Subtyping Patients with Cushing Syndrome

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BACKGROUND: Diagnosis of Cushing syndrome requires a multistep process that includes verification of hypercortisolism followed by identification of the cause of adrenocortical hyperfunction. This study assessed whether pituitary, ectopic, and adrenal subtypes of Cushing syndrome were characterized by distinct plasma steroid profiles that might assist diagnosis.

METHODS: In this retrospective cross-sectional study, mass spectrometric measurements of a panel of 15 plasma steroids were applied to 222 patients tested for Cushing syndrome. Disease was excluded in 138 and confirmed in 51 patients with pituitary Cushing syndrome, 12 with ectopic adrenocorticotropin secretion, and 21 with adrenal disease. Another 277 age- and sex-matched hypertensive and normotensive volunteers were included for comparison.

RESULTS: Compared with patients without disease, the largest increases in plasma steroids among patients with Cushing syndrome were observed for 11-deoxycortisol (289%), 21-deoxycortisol (150%), 11-deoxycorticosterone (133%), corticosterone (124%), and cortisol (122%). Patients with ectopic disease showed the most prominent increases, but there was considerable variation for other steroids according to subtype. Patients with adrenal disease had the lowest concentrations of androgens, whereas those with ectopic and pituitary disease showed the lowest concentrations of aldosterone. Plasma 18-oxocortisol was particularly low in ectopic disease. With the use of 10 selected steroids, subjects with and without different Cushing syndrome subtypes could be discriminated nearly as closely as with the use of salivary and urinary free cortisol, dexamethasone-suppressed cortisol, and plasma adrenocorticotropin (9.5% vs 5.8% misclassification).

CONCLUSIONS: Patients with different subtypes of Cushing syndrome show distinctive plasma steroid profiles that may offer a supplementary single-test alternative for screening purposes.

Hypercortisolism in endogenous Cushing syndrome (CS) is a condition associated with poor quality of life and high morbidity that if left untreated carries a median survival of <5 years *(1)*. Prompt diagnosis to ensure appropriately tailored therapy is therefore crucial for improved outcomes. This, however, is complicated by a multistep diagnostic process necessary not only to identify patients with CS but also the particular subtype.

According to current guidelines *(2)*, initial testing for CS should follow appropriate clinical suspicion and include at least 1 of the following tests: measurements of urinary or salivary free cortisol; a 1 mg overnight or 2 mg longer-duration dexamethasone suppression test (DST). Positive results are followed up by repeat or other tests of the screening combination to confirm disease. On confirmation, further testing is directed at distinguishing adrenocorticotropin (ACTH)-independent CS, due to autonomous cortisol secretion by an adrenal adenoma or bilateral adrenal nodular disease, from ACTH-dependent CS, usually reflecting an ACTH-secreting pituitary adenoma or more rarely due to ectopic ACTH secretion. For ACTH-dependent CS, a variety of further dynamic tests and imaging procedures are necessary to distinguish pituitary from ectopic subtypes and locate the source of ACTH or in occasional cases corticotropin-releasing hormone.

Although several early studies documented the use of plasma or urinary multisteroid profiles for character-

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Received September 28, 2017; accepted November 6, 2017.
Previously published online at DOI: 10.1373/clinchem.2017.282582

[6] Nonstandard abbreviations: CS, Cushing syndrome; DST, dexamethasone suppression test; ACTH, adrenocorticotropin; DHEA, dehydroepiandrosterone; DHEA-SO_4, dehydroepiandrosterone sulfate; ROC, receiver-operating characteristic.

Table 1. Demographic patient data and routine biochemical test results for patients screened for CS according to diagnosis.

		Patients tested for CS			
Diagnosis	Reference	Excluded	Adrenal	Pituitary	Ectopic
Demographics					
N	277	138	21	51	12
Sex (M/F)	99/178	49/89	3/18	16/35	5/7
Age (years)[a]	47 (18–81)	46 (16–80)	47 (17–72)	48 (17–78)	53 (26–75)
Routine Biochemistry					
Urine free cortisol (μg/24 h)[a]		143 (4–530)	603[b] (83–921)	714[b] (167–2812)	997[b,c] (218–16 078)
Salivary cortisol (ng/mL)[a]		1.1 (0.1–14.9)	3.9[b] (0.3–11.8)	9.8[b,c] (1.4–771)	29.8[b,c,d] (2.8–750)
ACTH (pg/mL)[a]		17 (1–244)	4[b] (2–35)	47[b,c] (9–206)	185[b,c,d] (11–847)
DX Serum cortisol (μg/dL[a])		1.1 (0.5–2.0)	14[b] (2.7–23.9)	14.4[b] (2.3–69.4)	37.7[b] (7.2–63.0)

[a]Data for age and routine biochemistry are shown as medians and ranges. ACTH, adrenocorticotropin; DX, dexamethasone.
[b]$P < 0.05$, different from excluded.
[c]$P < 0.02$ pituitary or ectopic different from adrenal.
[d]$P < 0.05$ ectopic different from pituitary.

izing patients with CS, the methods involved cumbersome paper or liquid chromatography and immunoassay procedures *(3–5)* or gas chromatography following derivatization *(6, 7)* and have not enjoyed continuing use. Nevertheless, several of these reports indicated distinct steroid profiles that could be useful for differential diagnosis of CS *(3–5)*. More recently LC-MS/MS has come to the forefront for not only routine measurements of serum, urinary free, or salivary cortisol *(8–10)* but also multisteroid profiling in disorders of adrenocortical function such as congenital adrenal hyperplasia *(11, 12)*, adrenal insufficiency *(12, 13)*, and primary aldosteronism *(14, 15)*. There has also been 1 report on the application of LC-MS/MS–based multisteroid profiling in subclinical CS *(16)* and 2 other recent reports directed to small series of patients with adrenocortical carcinoma and CS *(17, 18)*.

On the basis of the above considerations and following promising findings of early studies examining urinary and serum steroid metabolomes in CS *(3–7)*, we hypothesized that the 3 main subtypes of CS would show differences in plasma steroid profiles that might prove useful for disease identification and stratification. To address this hypothesis, we employed a newly developed LC-MS/MS method to measure 15 adrenal steroids in single plasma samples collected from 222 patients tested for CS, among whom disease was confirmed in 84 patients. Disease was excluded in the other 138 patients by conventional diagnostic testing. For comparative purposes a further 277 age- and sex-matched normotensive and hypertensive volunteers were selected from a previously described reference population *(19)*.

Materials and methods

SUBJECTS

We performed a bicentric cross-sectional study (as part of the German Cushing Registry) analyzing 222 study participants tested for CS at the Ludwig Maximilians Universität München (n = 161) and the University Hospital Dresden (n = 61). Details of the German Cushing Registry have been described elsewhere *(20)*. CS was confirmed or excluded on the basis of results of conventional diagnostic testing and follow-up (Table 1). Patients with subclinical hypercortisolism, defined by the absence of typical signs and symptoms of CS in the presence of abnormal biochemical test results for hypercortisolism, were excluded from the analysis. The 277 normotensive and hypertensive volunteers included for comparative purposes were selected on the basis of a match for age and sex from a larger population of 525 volunteers (all from Dresden) used for establishing age- and sex-specific reference intervals for each of the steroids of the plasma panel *(19)*. Subjects provided written informed consent under protocols approved by the local ethics committee at each center.

DIAGNOSIS OF CS

Diagnosis of CS followed current guidelines *(2)*. In short, initial screening was based on the presence of relevant clinical features and biochemical confirmation through the following screening tests: increased 24-h urinary free cortisol according to the upper cutoffs of reference intervals; loss of diurnal circadian rhythm in salivary cortisol with midnight salivary cortisol concentrations

exceeding 1.5 ng/mL; and lack of suppression of serum cortisol (fasting serum cortisol >1.8 μg/dL) with use of the low-dose DST with 1 mg overnight or 2 mg over 2 days.

Subsequent diagnosis of ACTH-dependent and independent CS and identification among the former group of pituitary vs ectopic ACTH-secreting tumors was based on various second-tier tests and imaging procedures carried out according to specific findings among individual patients as outlined elsewhere *(21)*. In total, 51 patients were identified with pituitary disease, 12 with ectopic disease, and 21 with adrenal disease (Table 1). Among the latter group, 17 had unilateral adenomas and 4 had bilateral micronodular or macronodular hyperplasia. Surgical and pathological confirmation of disease, with correction of hypercortisolism, was achieved in 79 (94%) patients. Three patients remained unoperated, and 2 were treated medically. Diagnosis of a corticotroph adenoma was confirmed later in 1 patient at autopsy.

Exclusion of CS was established by repeated biochemical assessments and clinical follow-up after 3–12 months. A repeatedly negative low-dose DST result (fasting serum cortisol <1.8 μg/dL) and absent clinical evidence of disease on follow-up represented the primary final criteria for exclusion of CS. Patients with continuing clinical suspicion and positive results of the DST, including patients with indications of subclinical CS, were excluded from analysis.

PLASMA STEROID PROFILING

All blood samples for plasma steroid profiling were collected in the morning (08:00–11:00 AM) into blood tubes containing lithium heparin or EDTA. Separated plasma was stored at −80 °C until the steroid profile was analyzed by LC-MS/MS. The 15-adrenal steroid panel included cortisol, 11-deoxycortisol, 21-deoxycortisol, corticosterone, 11-deoxycorticosterone, aldosterone, 18-oxocortisol, 18-hydroxycortisol, cortisone, progesterone, 17-hydroxyprogesterone, pregnenolone, androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEA-SO_4). Full details of the method, including assay performance characteristics, are described elsewhere *(22)*.

STATISTICAL ANALYSES

Statistical analyses used the JMP statistics software package (SAS Institute). The Kruskal–Wallis and the Steel Dwass all-pairs tests were used for nonparametric comparisons involving multiple groups. Spearman's rank correlation coefficient was used to assess significance of relationships. Significance was defined as $P < 0.05$. For parametric multivariate analyses, all data were logarithmically transformed before analyses. Because many of the steroids in the plasma panel show sex-specific differences and highly dynamic changes according to age, data for plasma steroids shown in figures were normalized according to age- and/or sex-specific upper cutoffs of reference intervals as established elsewhere *(19)*. Receiver-operating characteristic (ROC) curves for distinguishing patients with and without CS were constructed by logistic regression. Stepwise regression was used to select a minimal panel of the most useful steroids for diagnosis. Discriminant analysis with stepwise variable selection was further used to assess how combinations of plasma steroids could be used to distinguish various CS subtypes. Data from the above analyses were compared to results of diagnostic testing with use of routine tests.

Results

STEROID PROFILES

Patients in whom CS was suspected and excluded had higher plasma concentrations of cortisol ($P = 0.0023$), 18-oxocortisol ($P < 0.0001$), and 18-hydroxycortisol ($P < 0.0001$) than the reference population; the remaining 12 steroids showed no differences between the 2 groups without CS (Table 2). Among the 15 steroids in the panel, 11-deoxycortisol and 11-deoxycorticosterone were the only 2 that were consistently higher ($P < 0.0001$) in all subtypes of CS than in groups without CS. While patients with pituitary and ectopic subtypes had higher ($P < 0.0001$) plasma concentrations of cortisol than the 2 groups without CS, for patients with adrenal CS the difference was only significant ($P = 0.0051$) compared to the reference group. Similarly, compared to the 2 groups without CS, patients with pituitary and ectopic subtypes of CS had higher plasma concentrations of 21-deoxycortisol (pituitary vs both groups, $P < 0.0001$; ectopic vs reference, $P = 0.0004$; ectopic vs rule-out, $P = 0.0010$), corticosterone (pituitary vs both groups, $P < 0.0001$; ectopic vs reference, $P < 0.0001$; ectopic vs rule-out, $P = 0.0004$), and androstenedione (pituitary vs both groups, $P < 0.0001$; ectopic vs reference, $P = 0.0007$; ectopic vs rule-out, $P = 0.0053$), whereas there were no differences for those 3 steroids among patients with adrenal CS and the 2 groups without CS.

Similar to results for salivary and urinary free cortisol (Table 1), patients with ectopic disease had the largest increases of plasma glucocorticoids (Table 2). Plasma concentrations of cortisol among patients with ectopic ACTH secretion were higher than in patients with adrenal ($P = 0.0009$) and pituitary CS ($P = 0.0047$). Likewise, patients with ectopic disease had higher plasma concentrations of 11-deoxycortisol ($P = 0.0040$) and 11-deoxycorticosterone ($P = 0.0019$) than patients with pituitary CS, and higher ($P = 0.0194$) corticosterone concentrations than patients with adrenal CS.

Plasma concentrations of several other steroids showed both distinctive and divergent differences among different groups of CS patients compared to those with-

Table 2. Plasma concentrations of 15 adrenal steroids in patients with adrenal, pituitary, and ectopic CS compared to the reference population and patients in whom CS was suspected and excluded by routine biochemistry (see Table 1).

		Tested for CS			
Steroid	Reference	Excluded	Adrenal	Pituitary	Ectopic
Cortisol	87 (10–308)	107[a] (5–419)	163[a] (30–266)	205[a,b,c] (21–444)	371[a,b,c,d] (141–1362)
11-Deoxycortisol	0.14 (0.03–1.82)	0.15 (0.01–0.86)	0.50[a,b] (0.08–5.33)	0.48[a,b] (0.05–3.63)	2.00[a,b,d] (0.30–34.1)
21-Deoxycortisol	0.01 (0.00–0.76)	0.01 (0.00–0.23)	0.01 (0.00–2.50)	0.03[a,b] (0.00–0.30)	0.04[a,b] (0.01–0.13)
Cortisone	17.2 (1.2–32.3)	18.9 (1.1–35.8)	17.8 (4.5–31.3)	21.3[a,b] (2.5–34.8)	23.2 (12.0–34.7)
Corticosterone	1.58 (0.17–32.00)	1.69 (0.07–16.90)	2.2 (0.49–11.45)	3.73[a,b] (0.23–16.90)	8.78[a,b,c] (1.61–30.30)
11-Deoxycorticosterone	0.03 (0.00–0.37)	0.03 (0.00–0.12)	0.09[a,b] (0.02–0.61)	0.05[a,b,c] (0.00–0.33)	0.19[a,b,d] (0.02–4.37)
Aldosterone	0.05 (0.00–0.27)	0.05 (0.00–0.67)	0.06 (0.01–1.09)	0.03[a,b] (0.01–0.31)	0.01[b,c] (0.01–0.17)
18-Oxocortisol	0.008 (0.001–0.09)	0.013[a] (0.003–0.33)	0.014[a] (0.002–0.50)	0.012 (0.01–0.07)	0.002[b,c] (0.002–0.03)
18-Hydroxycortisol	0.59 (0.03–2.59)	0.78[a] (0.02–5.86)	0.76 (0.22–4.20)	1.05[a] (0.07–5.32)	1.31[a] (0.26–3.44)
Pregnenolone	1.44 (0.18–11.20)	1.38 (0.04–10.50)	1.85 (0.15–6.06)	1.84[b] (0.15–5.58)	2.43 (0.15–10.26)
17-Hydroxyprogesterone	0.41 (0.04–2.74)	0.44 (0.06–1.90)	0.35 (0.04–6.43)	0.48 (0.04–2.36)	0.96[b] (0.20–6.83)
Progesterone	0.09 (0.01–26.10)	0.09 (0.04–13.40)	0.09 (0.01–11.16)	0.08 (0.01–10.11)	0.16[d] (0.01–11.16)
Androstenedione	0.87 (0.21–3.94)	0.97 (0.12–3.70)	0.49 (0.06–4.05)	1.70[a,b,c] (0.11–7.12)	1.99[a,b,c] (0.63–30.30)
DHEA	2.62 (0.40–16.90)	2.58 (0.03–25.9)	0.62[a,b] (0.04–11.55)	3.89[c] (0.03–13.00)	1.76[c] (0.52–18.90)
DHEA-SO_4	1420 (135–6140)	1366 (25–4440)	238[a,b] (5–6325)	2120[a,b,c] (33–5590)	1598[c] (29–5260)

All plasma concentrations are shown as medians and ranges in ng/mL.
[a] $P < 0.05$, different from reference.
[b] $P < 0.05$, different from excluded.
[c] $P < 0.05$ pituitary or ectopic different from adrenal.
[d] $P < 0.05$ ectopic different from pituitary.

out CS. In particular, plasma aldosterone was lower in patients with pituitary CS ($P = 0.0040$) and ectopic ACTH secretion ($P = 0.0230$) than in those in whom CS was excluded (Table 2). Patients with ectopic disease also had lower ($P = 0.0374$) plasma aldosterone concentrations than patients with adrenal CS. Similarly, plasma 18-oxocortisol concentrations were lower in patients with ectopic ACTH secretion than in those with adrenal CS ($P = 0.0427$) and patients in whom CS was excluded ($P = 0.0021$).

The adrenal androgens showed an opposite pattern to that of aldosterone and oxocortisol, with lower concentrations in adrenal CS than in pituitary CS (androstenedione, $P = 0.0027$; DHEA, $P < 0.0001$; DHEA-SO_4, $P < 0.0001$) and ectopic ACTH-secreting tumors (androstenedione, $P = 0.0106$; DHEA, $P = 0.0273$; DHEA-SO_4, $P = 0.0380$). Plasma DHEA and DHEA-SO_4 concentrations were also lower ($P < 0.0001$) in the adrenal CS group than in groups without CS. In contrast, DHEA-SO_4 was higher in patients with pituitary CS than in the reference group ($P = 0.0048$) and patients in whom CS was excluded ($P = 0.0044$). Differences among patient groups for cortisone, 18-hydroxycortisol, pregnenolone, 17-hydroxyprogesterone, and progesterone were more isolated and less pronounced than for other steroids.

Overall the various steroids in the panel showed distinctive patterns of differences between patients with ACTH-independent and dependent forms of CS, including among the latter group differences between patients with pituitary and ectopic subtypes (Fig. 1). In part, the differences between the 2 ACTH-dependent subtypes appeared to reflect the more pronounced nature of disease in ectopic than pituitary disease (Table 1). Nevertheless, the more pronounced nature of disease in ectopic than pituitary subtypes was not reflected by all steroids in the panel, in particular 18-oxocortisol, DHEA, and DHEA-SO_4.

RELATIONSHIPS OF STEROIDS AND ACTH

Among patients tested for CS, ACTH showed significant positive relationships with all steroids except aldosterone, 18-oxocortisol, and progesterone (Table 3). For aldosterone there was a significant negative relationship with ACTH ($r_s = -0.2626$, $P < 0.0001$). Apart from aldosterone and 18-oxocortisol, the 13 other steroids of the panel showed significant positive relationships for 92% of all steroid comparisons. Aldosterone showed a strong positive relationship with 18-oxocortisol ($r_s = 0.6422$, $P < 0.0001$), weaker relationships with 18-hydroxycortisol ($r_s = 0.1537$, $P = 0.0006$) and corticosterone ($r_s = 0.1332$, $P = 0.0029$), and considerably

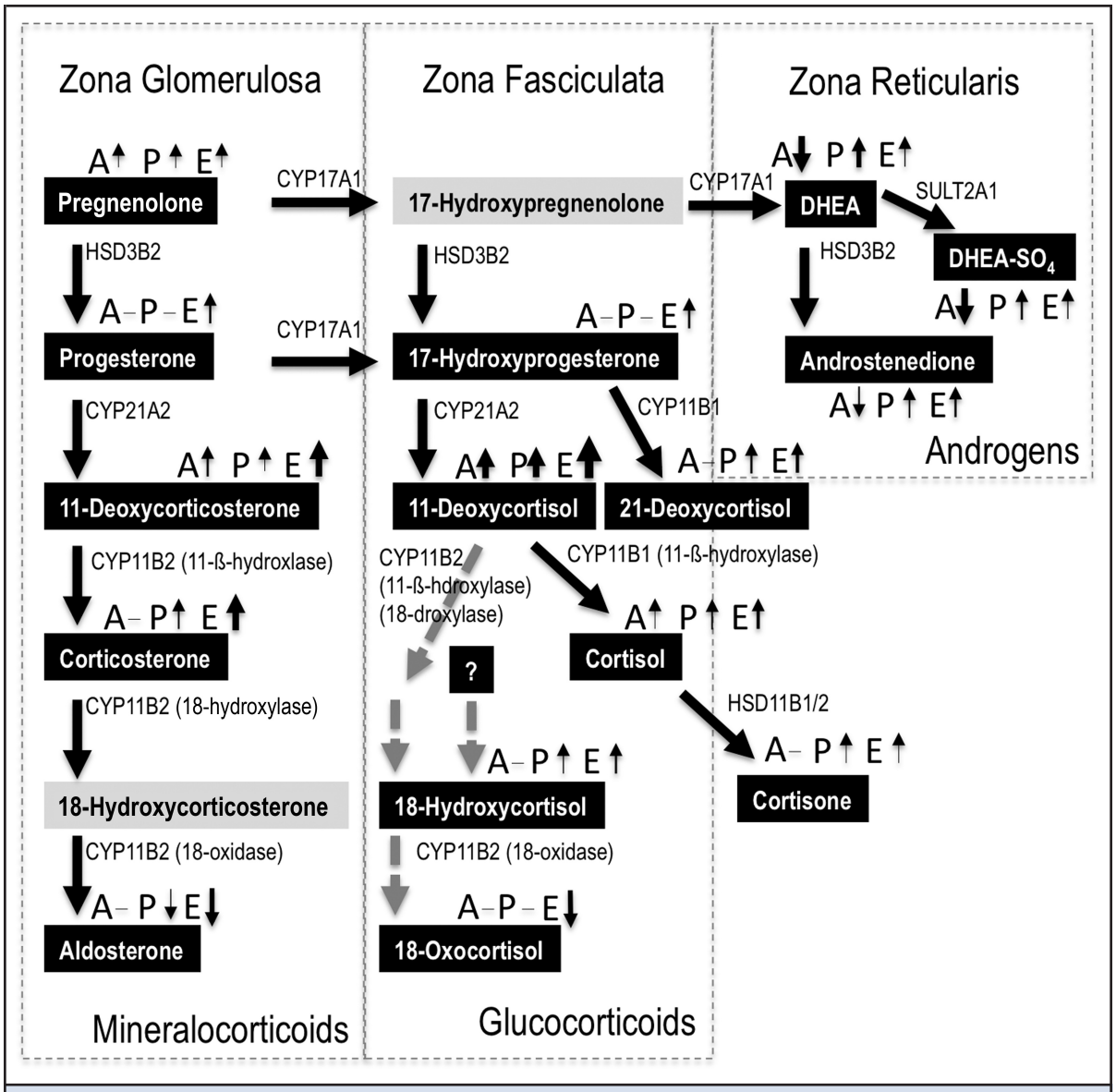


Fig. 1. Schematic diagram showing the biosynthetic pathways for the 15 adrenal steroids in the panel (black background) and 2 other steroids (light background) not included in the panel.

Also shown are the enzymes catalyzing each biosynthetic step. The exact pathways of synthesis shown by the gray dashed arrows for the hybrid steroids, 18-hydroxycortisol and 18-oxocortisol, are incompletely established; however, synthesis of 18-oxocortisol is believed to depend on metabolism of the glucocorticoid, 11-deoxycortisol, by aldose synthase (CYP11B2), an enzyme localized mainly to the zona glomerulosa and normally responsible in a multistep process for conversion of 11-deoxycorticosterone to aldosterone. The relative increases or decreases for each plasma steroid compared to the reference population are shown for adrenal (A), pituitary (P), and ectopic (E) subtypes of CS, with extents of increase or decrease indicated by the size of arrows or lack of difference by a dash (–).

weaker (4/11) or nonexistent (7/11) relationships with the other 11 steroids of the panel.

DIAGNOSIS OF CS

Urinary and salivary free cortisol showed considerable overlap among patients with and without CS (Fig. 2, A and B), as did all 15 steroids in the plasma panel, including the 3 steroids (11-deoxycortisol, 11-deoxycorticosterone, and cortisol) that provided the most consistent differences among patient groups (Fig. 2, D–F). There was no overlap for serum cortisol after dexamethasone (Fig. 2C). According to the upper cutoffs of reference intervals for routine tests,

Table 3. Spearman correlation coefficients (r_s) and levels of significance for relationships of plasma ACTH with steroids of the plasma panel.

Steroid	r_s value	*P*-value
Cortisol[a]	0.4852	<0.0001
11-Deoxycortisol[a]	0.5733	<0.0001
21-Deoxycortisol[a]	0.3275	<0.0001
Cortisone[a]	0.2914	<0.0001
Corticosterone[a]	0.4988	<0.0001
11-Deoxycorticosterone[a]	0.3496	<0.0001
Aldosterone	−0.2626	<0.0001
18-Oxocortisol	−0.1029	0.1281
18-Hydroxycortisol[a]	0.3070	<0.0001
Pregnenolone[a]	0.2084	0.0031
17-Hydroxyprogesterone[a]	0.1868	0.0082
Progesterone[a]	0.0537	0.4511
Androstenedione	0.3940	<0.0001
DHEA	0.3477	<0.0001
DHEA-SO_4	0.3846	<0.0001

[a]Analysis for these steroids omits data for adrenal CS patients.

values for diagnostic sensitivity and specificity were 94.5% and 65.3% for salivary free cortisol, 97.4% and 50.8% for urinary free cortisol, and 100% and 97% for the DST. For the steroid panel, upper cutoffs were individualized for age and/or sex according to the 97.5 percentiles of our previously characterized reference population *(19)*, yielding respective diagnostic sensitivities and specificities of 61% and 99% for plasma 11-deoxycortisol, 30% and 99% for plasma 11-deoxycorticosterone, and 27% and 95% for plasma cortisol.

Areas under ROC curves indicated higher ($P < 0.001$) diagnostic performance of the DST than any single measurement or measurement combination (Fig. 2, G and H). Salivary and urinary free cortisol exhibited similar diagnostic performance and both showed higher performance than plasma cortisol ($P = 0.0003$). Urinary free cortisol also showed higher diagnostic performance than 11-deoxycortisol ($P = 0.0208$), whereas there was no difference between salivary cortisol and 11-deoxycortisol ($P = 0.0891$). With use of stepwise regression analyses, a combination of 7 steroids (11-deoxycortisol, 11-deoxcorticosterone, cortisol, aldosterone, 21-deoxycortisol, DHEA, and DHEA-SO_4) was identified to provide optimal diagnostic performance for distinguishing patients with and without CS (Fig. 2H). The area under the ROC curve for the 7-steroid combination did not differ from areas for salivary or urinary free cortisol and was higher than for any other single plasma steroid in the panel (11-deoxycortisol, $P = 0.0408$; cortisol, $P = 0.0005$; all 13 other steroids, $P < 0.0001$). At the optimal point in the ROC curve, the 7-steroid panel offered a diagnostic sensitivity of 87% at a specificity of 89%.

DISCRIMINATION OF CS SUBTYPES

Complete results for routine measurements of salivary and urinary free cortisol combined with the DST and measurements of plasma ACTH were available in 173 of all 222 patients tested for CS. By discriminant analysis, use of the combination of 4 routine measurements correctly classified all patients with adrenal CS among the total of 173 patients in whom the 4 routine measurements were available (Fig. 3, A–C). Among that group of 173 patients, all of those in whom CS was excluded were also correctly classified, and canonical plots indicated no overlap between patients with ectopic or adrenal subtypes from those in whom CS was excluded (Fig. 3A). Areas under ROC curves varied from 0.9786 for ectopic disease to 0.9987 for adrenal CS (Fig. 3B). Among all 173 patients, only 5.8% were not correctly classified by the combination of all 4 routine measurements, this mainly reflecting overlap for patients with pituitary and ectopic subtypes among whom 80% were correctly identified according to discriminant scores (Fig. 3C). Omitting either salivary or urinary free cortisol from the 4-test combination only slightly increased the misclassification rate (7.3% and 9.0%). In contrast, omitting ACTH or the DST more substantially increased misclassification to 21.4% and 13.7%, respectively, with the former reflecting poorer discrimination of adrenal from pituitary and ectopic subtypes and the latter poorer discrimination of patients with and without CS.

By use of stepwise variable selection, a combination of 10 plasma steroids (11-deoxycortisol, cortisol, cortisone, corticosterone, 11-deoxycorticosterone, androstenedione, 18-oxocortisol, DHEA, DHEA-SO_4, and aldosterone) in the panel were found to provide optimal discrimination of the 3 subtypes of CS (Fig. 3, D–F). Although canonical plots indicated more overlap with use of the 10-steroid combination than with use of all 4 routine tests (Fig. 3, A and B), areas under ROC curves were nevertheless similarly large, ranging from 0.9653 for patients in whom CS was excluded to 1.000 for patients with ectopic CS (Fig. 3E).

By use of the 10-steroid combination, all patients with ectopic ACTH secretion were correctly classified, only 1 patient with adrenal CS was incorrectly classified, and most of the patients with pituitary CS were correctly classified (Fig. 3F). The 10-steroid combination resulted in incorrect classification of 9.5% of all patients, this almost completely reflecting incomplete discrimination of patients with and without CS (Fig. 3F). With addition of the DST to the 10-steroid panel, misclassification fell

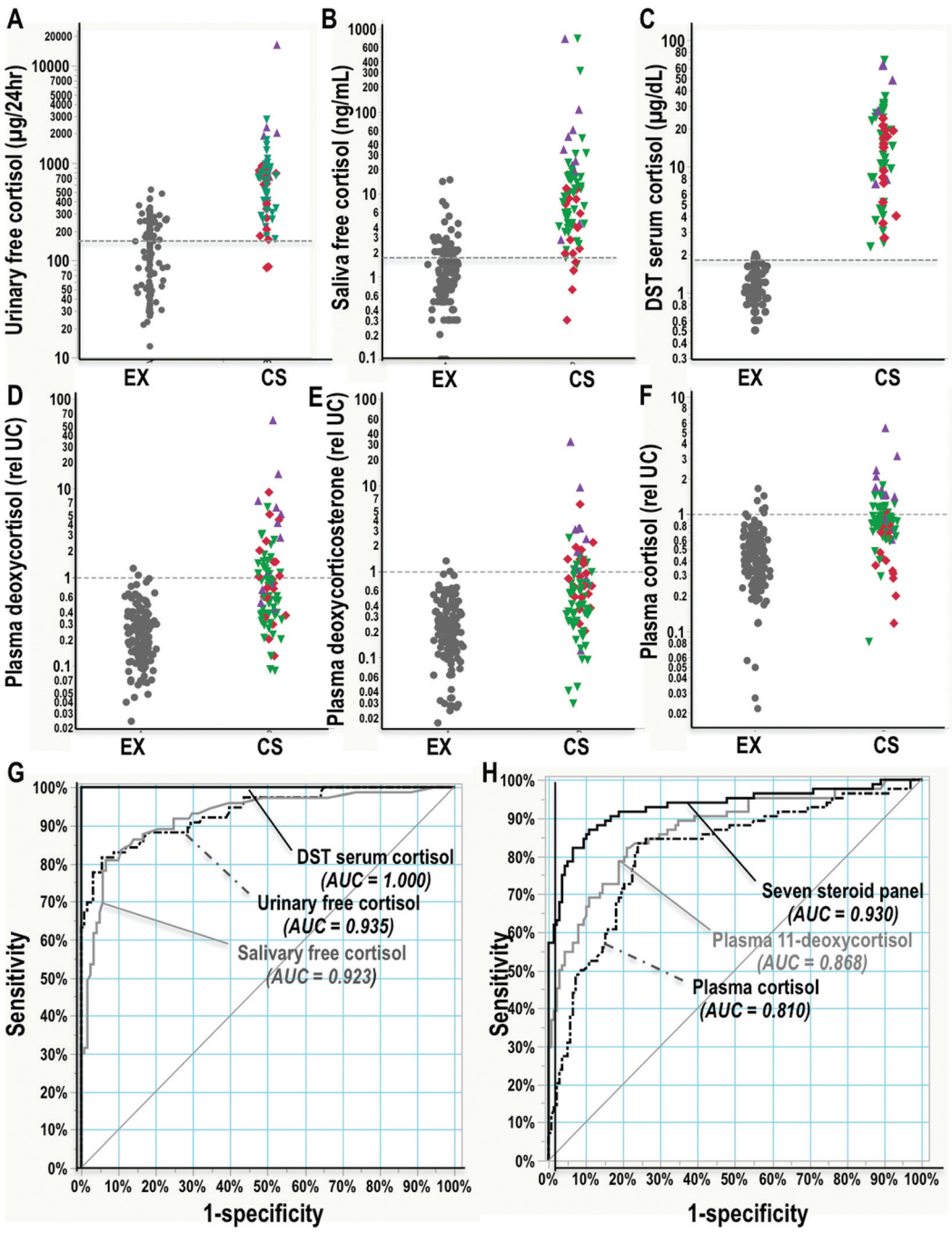
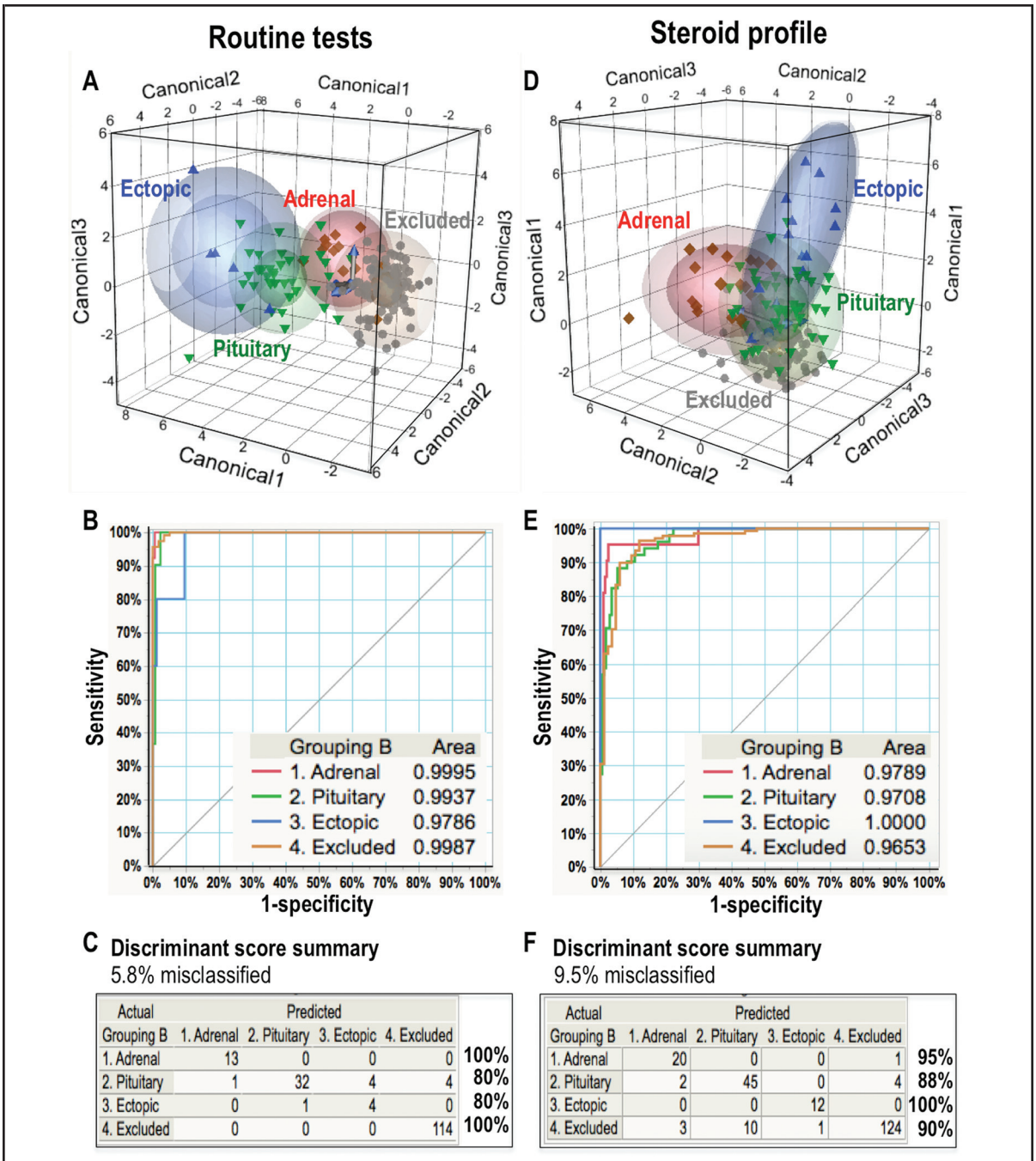


Fig. 2. Results of routine diagnostic tests (A, B, C, G) and selected steroids (D, E, F, H) of the 15-steroid panel in patients with CS (CS) and in patients in whom CS was excluded (EX).

Absolute values for urinary free cortisol (A), salivary free cortisol (B), and DST plasma cortisol concentrations (C) are shown with respect to upper cutoffs of reference intervals used in routine clinical practice. Values for the 3 selected steroids, 11-deoxycortisol (D), 11-deoxycorticosterone (E), and cortisol (F), of the 15-steroid panel are shown as a proportion of sex- and age-specific upper cutoffs (rel UC) for which upper cutoffs are designated with a value of 1. Among the different patients with and without CS, the 4 groups are distinguished by different symbols (ectopic CS ▲, pituitary CS ▼, adrenal CS ◆, CS excluded ●). ROC curves are shown in panel G for the 3 routine screening tests and in panel H for plasma cortisol, 11-deoxycortisol, and a selection of 7 steroids of the steroid panel that provided optimal discrimination of patients with and without CS.



Actual Grouping B	Predicted 1. Adrenal	2. Pituitary	3. Ectopic	4. Excluded	
1. Adrenal	13	0	0	0	100%
2. Pituitary	1	32	4	4	80%
3. Ectopic	0	1	4	0	80%
4. Excluded	0	0	0	114	100%

Actual Grouping B	Predicted 1. Adrenal	2. Pituitary	3. Ectopic	4. Excluded	
1. Adrenal	20	0	0	1	95%
2. Pituitary	2	45	0	4	88%
3. Ectopic	0	0	12	0	100%
4. Excluded	3	10	1	124	90%

Fig. 3. Results of discriminant analyses for use of routine diagnostic tests (A, B, C) compared to 10 steroids of the steroid panel (D, E, F) that provided optimal discrimination of the 4 patient groups (ectopic CS ▲, pituitary CS ▼, adrenal CS ◆, CS excluded ●).

Three-dimensional canonical plots are shown in panels A and D; ROC curves with areas under curves are shown in panels B and C; whereas predicted versus actual groupings according to discriminant analyses are shown in panels C and F. Routine diagnostic tests included measurements of salivary and urinary free cortisol, DST plasma cortisol, and ACTH. Steroids for the selected steroid profile included 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, cortisol, corticosterone, DHEA, DHEA-SO_4, androstenedione, aldosterone, and 18-oxocortisol. Note that for the analyses of routine clinical tests, complete results for all 4 tests were only available in 173 of the 222 patients.

to 4.9% and no patient with CS was incorrectly designated not to have CS.

Discussion

This study using simultaneous LC-MS/MS measurements of 15 adrenal steroids in plasma establishes distinct steroid metabolome profiles that might be useful as a test for CS. Specimen preparation is simple, the entire panel takes 12 min to run, and the method has the potential for offering a single test for both screening of CS and initial subtype classification. Another advantage over other tests is that different selections of steroids from the panel may be used for other disorders of adrenal steroidogenesis, including primary aldosteronism, adrenocortical carcinoma, and congenital adrenal hyperplasia *(14, 15, 17)*, providing versatility of the method.

LC-MS/MS is rapidly gaining recognition as the method of choice for accurate measurements of specific steroids and other low molecular weight analytes used in the diagnosis of endocrine disorders. Applications of LC-MS/MS are now also being increasingly developed for targeted analyses of multiple analytes. Gas chromatography coupled to mass spectrometry also offers a useful analytical platform for multisteroid profiling in adrenal disorders *(23–26)*. However, these methods invariably involve labor-intensive sample processing, including the necessity of a derivatization step, and offer limited sample throughput due to extended chromatographic run times. Consequently, LC-MS/MS offers a preferable platform, particularly for routine clinical purposes *(27, 28)*.

Among the steroids examined, 11-deoxycortisol, the immediate precursor of cortisol, showed the strongest diagnostic signal for distinguishing patients with and without CS. Recently, plasma 11-deoxycortisol and its urinary metabolite, tetrahydro-11-deoxycortisol, were also shown to provide the best steroid biomarkers for adrenocortical carcinoma *(17, 18)*. In other studies, 11-deoxycortisol was superior to cortisol for identifying patients with adrenal insufficiency and indicating correct catheter positions during adrenal venous sampling *(13, 22, 29)*. Poor diagnostic utility of plasma cortisol compared to salivary or urinary free cortisol and other indices of glucocorticoid production, such as 11-deoxycortisol, may result both from the considerable amounts bound to cortisol-binding globulin as well as impacts of the cortisol-cortisone shunt *(30, 31)*. In the present study, diagnostic performance for 11-deoxycortisol was strengthened by addition of other steroids in a 7-steroid combination that together provided similar diagnostic performance to salivary free cortisol.

Although indicating some promise for diagnosis of CS, inspection of ROC curves for the various tests examined for distinguishing patients with and without CS indicates that the selected 7-steroid combination is unlikely to offer a single-test replacement for all 4 currently recommended screening tests, particularly the DST. Nevertheless, it should also be appreciated that the apparently exceptional diagnostic performance of the DST in the present analysis is misleading, since patients with positive DST results (i.e., a serum cortisol of 1.8 μg/dL) and no confirmation of CS were excluded from the analysis. Although a cutoff of 1.8 μg/dL for a post DST plasma cortisol is useful for identifying about 95% of patients with CS and excluding CS in almost all patients with lower values, this cutoff is associated with false-positive rates of around 20%, indicating inadequate specificity for excluding CS in all disease-free patients *(32, 33)*. Thus, the area under the ROC curve of 1.0 for the DST is a misleading artifact resulting from the necessity to examine plasma steroid profiles in well-defined populations of patients with and without CS.

Measurements of steroid profiles before and after the DST might offer improved diagnostic performance over single plasma cortisol measurements. In this case it would also be a simple matter to include measurements of plasma dexamethasone in the panel to further improve diagnostic performance *(34, 35)*. Increased diagnostic performance of the 7-steroid panel, above that for late-night salivary cortisol, might also be expected for blood sampling at night rather than in the morning. Nevertheless, the ease and simplicity of sampling saliva without need for an overnight hospital stay offers advantages for salivary over plasma measurements. With availability of more analytically sensitive mass spectrometers, it might become possible to examine steroid profiles in saliva or even hair *(36)*.

Our findings that the various subtypes of CS show distinctive steroid fingerprints support previous findings of different steroid profiles in ACTH-dependent and independent forms of CS *(4, 17, 18, 37)*. In particular, low urinary excretion of DHEA in adrenal CS compared to pituitary disease *(4)* was subsequently determined to extend to a similar pattern for serum DHEA-SO_4 *(37)*. Reduced plasma concentrations of DHEA and androstenedione have also been documented by LC-MS/MS measurements in patients with subclinical CS compared to age-matched controls *(16)*. More recently, 2 further independent studies confirmed lower plasma and urinary androgens in ACTH-independent CS than in ACTH-dependent forms of CS *(17, 18)*.

In contrast to the pattern for androgens, the much lower plasma concentrations of aldosterone in ACTH-dependent CS than in independent forms of CS is not clearly established in the literature. In one study, plasma concentrations of aldosterone were reported to be suppressed in pituitary CS *(38)*, whereas other reports yielded conflicting results *(39, 40)*. Possibly suppressed aldosterone may reflect reduced renin activity secondary to fluid and salt retention in the more severe forms of CS

associated with pituitary and ectopic compared to adrenal disease. Interestingly, 18-oxocortisol also showed low plasma concentrations confined to patients with ectopic CS, a finding that might reflect suppression of the last enzymatic step in conversion of 11-deoxycortisol to 18-oxocortisol by aldosterone synthase *(41)*.

Apart from the aforementioned differences in aldosterone, hybrid steroids, and adrenal androgens, the various groups were also characterized by differences in other steroids such as corticosterone and 11-deoxycorticosterone. Combined with 11-deoxycortisol, cortisol, and cortisone, these steroids provided a 10-steroid panel that identified the various subtypes of CS with near comparable effectiveness as the combination of all routine diagnostic tests. Although the routine test combination was expectedly more effective than the plasma steroid profile for distinguishing patients with ACTH-dependent and independent CS, the plasma steroid profile appeared to show heightened effectiveness for distinguishing ectopic from pituitary and adrenal disease. Furthermore, by combining plasma steroid profiles with the DST, discrimination was as effective as all 4 routine tests.

While mass spectrometric assays of the steroid metabolome are unlikely to offer a single test alternative to all currently recommended screening tests for CS, the plasma steroid panel may be useful as a supplementary single-test alternative to both screen for CS and guide second-tier testing for discrimination of the 3 main CS subtypes. In this way, use of the plasma steroid panel as an alternative to measurements of urinary and salivary free cortisol might offer a cost-effective approach for streamlining the diagnostic process by reducing the number of screening and follow-up tests to confirm or exclude CS, as well as decreasing the number of subsequent second-tier steps ahead of procedures to localize tumors within the adrenal, pituitary, or at sources of ectopic ACTH secretion.

The retrospective nature of the study, with reliance on routine tests to rule out CS, represents a limitation of the present analysis. Indeed, findings of slight but significant differences in plasma cortisol, 18-oxocortisol, and 18-hydroxycortisol between patients in whom CS was excluded and the reference group suggest that within the former group there may have been patients with subtle hypercortisolism who were not identified by routine tests. This and the need to exclude patients in whom CS could not be ruled out by the DST represent shortcomings ensuring that it could not be possible to determine any significant improvement in diagnostic performance of the plasma steroid metabolome compared to routine diagnostic tests. Inclusion of a validation cohort would also have been useful, underlying the importance of further prospective studies to confirm the results of the present series. With the above limitations aside, the study remains important for providing novel data about a disease for which there remains considerable need for more effective and efficient strategies for diagnosis and subtype classification.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: G. Eisenhofer, European Commission Horizon 2020 Program under grant agreement 633983-2 (ENSAT-HT); F. Beuschlein, the European Commission Horizon 2020 Program under grant agreement 633983-2 (ENSAT-HT); M. Reincke, the European Commission Horizon 2020 Program under grant agreement 633983-2 (ENSAT- HT), Else Kröner-Fresenius Stiftung (2012-A103 and 2015-A228), the European Research Council (Grant number 694913 [PAPA]).
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

Acknowledgment: The authors thank Denise Kaden for technical assistance, Katharina Langton, and Stephanie Zopp for assistance with clinical studies, as well as Drs. Christina Berr and Finn Strasding for help with patient management.

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